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DEVELOPMENT OF TAIL MUSCLE ACETYLCHOLINESTERASE IN ASCIDIAN EMBRYOS LACKING MITOCHONDRIAL LOCALIZATION AND SEGREGATION

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Ascidians (subphylum Urochordata; class Ascidiacea) develop a large number of mitochondria during oogenesis. At fertilization many of these become localized into a myoplasmic crescent and are subsequently segregated by the determinate cleavage mechanism into the muscle lineage cells of the developing larva. In some species colored yolk granules are associated with the mitochondria; they serve as visible markers of the mitochondrial localization and its segregation (Conklin, 1905; Berrill, 1929; Berg and Humphreys, 1960). Many different staining techniques have been used to establish that oviparous species have mitochondrial segregation even in the absence of a visible crescent (*e.g.*, Meves, 1913; Duesberg, 1915; Mancuso, 1952; Reverberi, 1956).

This obvious association of large numbers of mitochondria with the ascidian larval muscle cell lineage has raised questions about whether the crescent mitochondria are (a) permissive, (b) selective, or even (c) instructive of muscle differentiation. An equally important question is whether segregation of the cytoplasmic determinants responsible for muscle differentiation (Whittaker, 1973) is linked directly to the mechanisms that localize and segregate mitochondria into the muscle cells. The results of various observations and experiments that have attempted to answer these questions prove to be contradictory. The suggestion has therefore persisted in some of the review literature that mitochondrial localizations are causally related to muscle cell determination and development (Brachet, 1960, 1974; Minganti, 1961; Reverberi, 1961, 1971).

Conklin (1931) displaced mitochondria by centrifugation of unfertilized ascidian eggs and concluded from his results that localizations of mitochondria were not the cause of muscle determination. Mitochondria could be driven out of the finely granular plasm in which they were found without preventing the formation of muscle during subsequent development. When the plasm itself was displaced, the larval muscles were also displaced. Tung, Ku, and Tung (1941) noted that mitochondrial masses moved by centrifugation to neural and ectodermal regions did not cause these cells to develop myofibrillae.

The first contradictory evidence came from centrifugation experiments by Ries (1939) who found that displacement of the indophenol oxidase-containing plasm of the ascidian egg resulted in a change in muscle development; he did not know at the time that this enzyme was a mitochondrial oxidase. La Spina (1958) also showed that mitochondrial displacement resulted in some abnormalities of muscle development. Direct interference with mitochondrial function, using inhibitors of the mitochondrial enzymes, resulted in the development of embryos markedly

deficient in muscle structures (de Vincentiis, 1956; Reverberi, 1957). Recently, Bell and Holland (1974) have found by microsurgically dividing centrifugally stratified eggs in various orientations and fertilizing the fragments that a certain limited number of mitochondria appear to be necessary for muscle differentiation. Their data also confirm the possibility that mitochondrial localizations might be causally related to muscle differentiation.

One of the difficulties with these various traumatic and disruptive experimental interventions is that they cause severe abnormalities in the embryos. Results are subject to considerable selection and interpretation by the investigator. In nature, however, a situation occurs in which the question can be clearly resolved in a qualitative way. Certain ascidian species have secondarily evolved anural larvae which no longer develop the larval tail (Berrill, 1931). One of these species, *Molgula arenata,* differentiates larval tail muscle up to the point of producing histospecific muscle acetylcholinesterase (Whittaker, 1979). This species does not localize mitochondria or segregate them into the muscle lineage cells.

Materials and Methods

Materials

The specimens of *Molgula arenata* Stimpson used in this study were dredged from sand flats near Senator Shoal in northern Nantucket Sound at Cape Cod, June through November. Control observations were made on embryos of two urodele species: *Ciona intestinalis* (L.), obtained in the vicinity of Woods Hole, Massachusetts, and *Molgula occidentalis* Traustedt of the Florida Gulf, purchased from the Gulf Specimen Company, Panacea, Florida. These three species do not have a visibly colored mitochondrial crescent.

Gametes were obtained by techniques described previously (Whittaker, 1979). Embryos were cultured in filtered sea water at $18 \pm 0.1°$ C using a refrigerated constant temperature water bath. Since the time of first division is variable in *M. arenata* (Whittaker, 1979), development times are expressed as time after the 2-cell stage.

Histochemistry

Acetylcholinesterase was localized in embryos by the Karnovsky and Roots (1964) procedure after 2 to 3 min fixation in cold (5° C) 80% ethanol (Durante, 1956). Incubation was for 12 hr at 18° C. Various substrate and inhibitor controls for the identity of the enzyme are presented elsewhere (Whittaker, 1979).

Succinic dehydrogenase activity was detected by the standard technique for bound mitochondrial enzymes described by Pearse (1972) using sodium succinate as substrate and nitro blue tetrazolium as the electron acceptor. Cytochrome oxidase was localized by the diaminobenzidine reaction of Seligman, Karnovsky, Wasserkrug, and Hanker (1968) including 20 μg/ml Sigma C-40 catalase in the reaction medium to prevent peroxidase activity. The fixative used for both enzymes was Karnovsky's (1965) fixative, but with formaldehyde and glutaraldehyde each reduced to 1.5%, half of the originally recommended strength. Fixation was for 5 to 15 min at 5° C. Incubation time for each of the mitochondrial enzymes

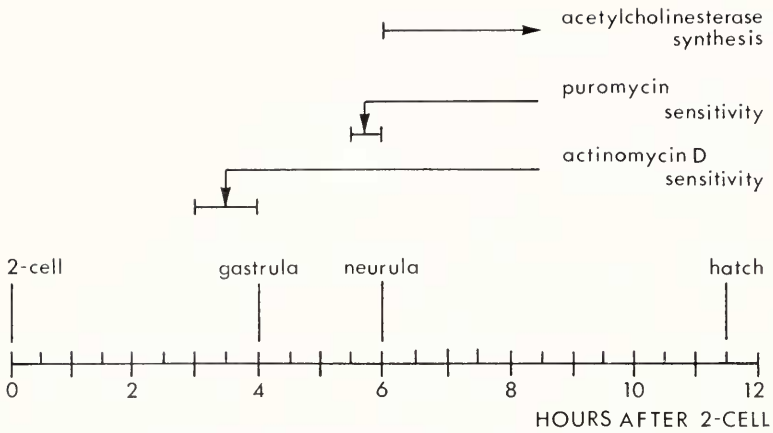


FIGURE 1. Time of various developmental stages and other events in specimens of *Molgula arenata* at 18° C.

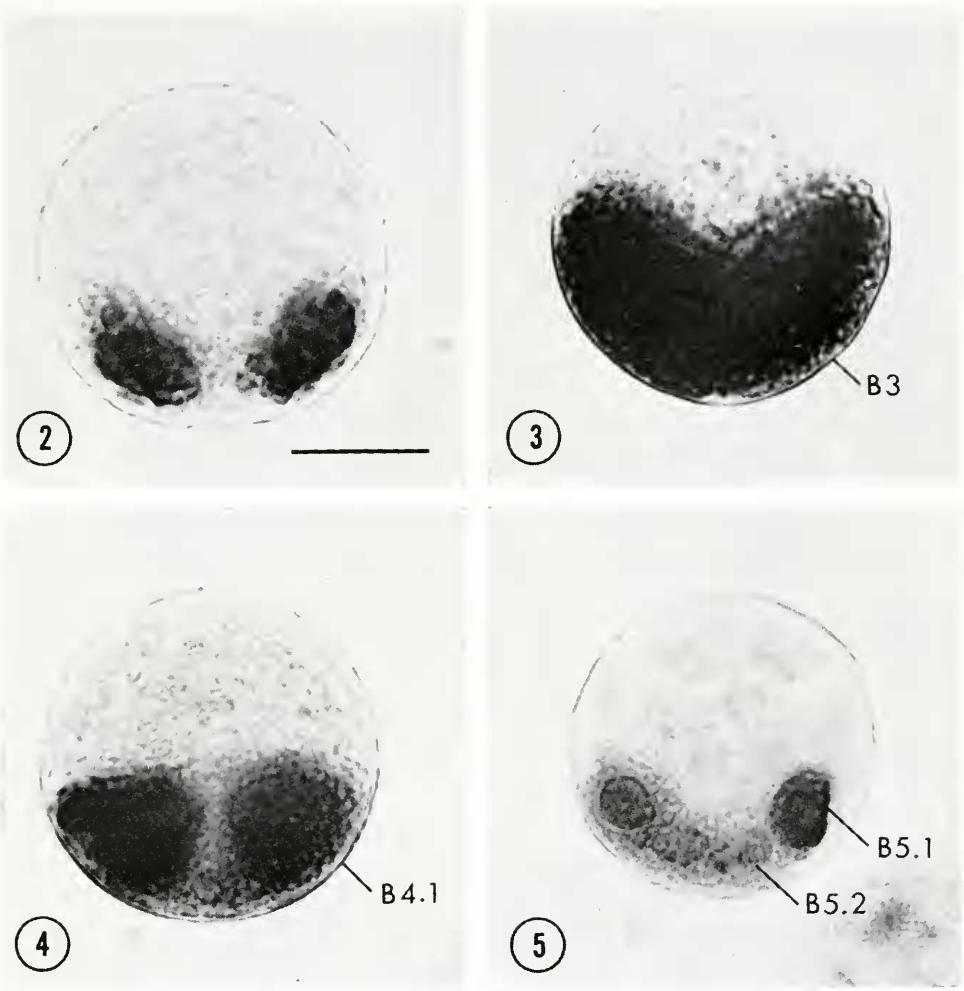
was 90 to 120 min at 22° C. Succinic dehydrogenase staining did not occur when sodium succinate was omitted from the incubation medium; the enzyme activity was not affected significantly by 10 mM sodium azide. Cytochrome oxidase activity did not occur in the presence of either 10 mM sodium azide or 10 mM potassium cyanide. Cytochrome c greatly enhances the sensitivity of the reaction but cytochrome oxidase gives a modest reaction with 3,3′-diaminobenzidine in the absence of substrate. Concentrations of the reaction products in both of these histochemical reactions appear to follow the Beer-Lambert law; color density is proportional to enzyme activity and therefore proportional to the numbers of mitochondria (Cabrini, Vinuales, and Itoiz, 1969; Marinos, 1978).

After the respective histochemical reactions the embryos were dehydrated in ethanol, cleared in xylene, and mounted in damar resin. The three enzyme procedures produced essentially permanent color reactions.

RESULTS

Development of acetylcholinesterase

Development of larval tail muscle acetylcholinesterase in *Molgula arenata* initially followed the same pattern found in urodele ascidian species: the enzyme became histochemically detectable at neurulation. Color first appeared at 6 hr after the 2-cell stage (Fig. 1) and enzyme activity accumulated gradually over the next few hours until a modest level of activity was attained (Fig. 2). In some larvae this activity reached a level as high as 20% of that found in the larvae of comparable urodele species of *Molgula*, but the mean activity found was 5 to 6% of the urodele level (Whittaker, 1979). Tail development was completely suppressed in *M. arenata* and except for acetylcholinesterase development presumptive muscle tissues did not otherwise develop beyond the early neurula stage; there was no obvious myofibrillar synthesis.



FIGURES 2–5. Embryos of *Molgula arenata* stained histochemically for acetylcholinesterase.

FIGURE 2. Hatched larva.

FIGURE 3. Embryo cleavage-arrested in cytochalasin B at the 4-cell stage and reacted for enzyme at 8 hr after the time of the 2-cell stage.

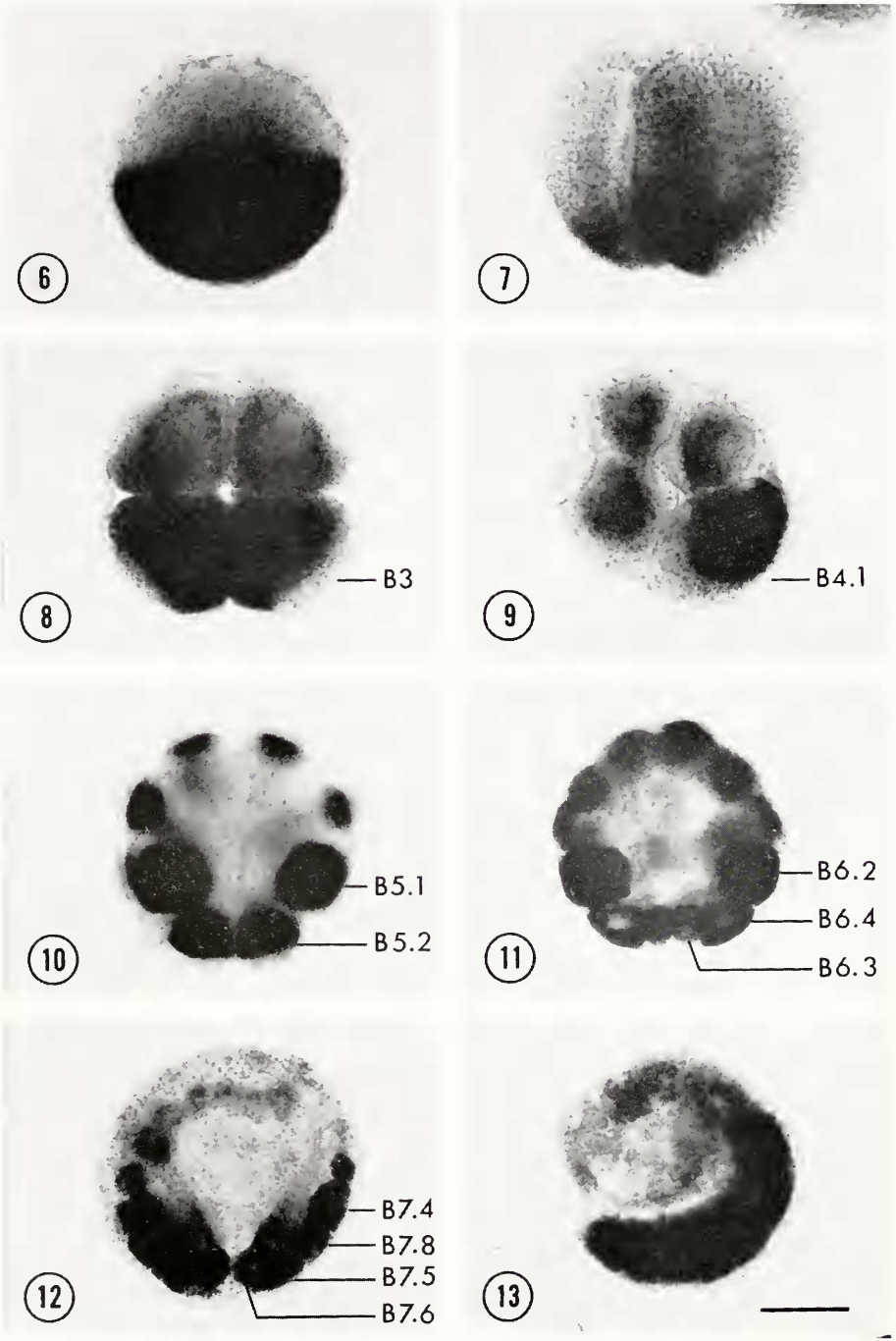
FIGURE 4. Embryo cleavage-arrested at the 8-cell stage and reacted for enzyme at 8 hr after the 2-cell stage.

FIGURE 5. Embryo cleavage-arrested at the 16-cell stage and reacted for enzyme at 8 hr after the 2-cell stage.

The muscle cell lineage designations are according to Ortolani (1955). All figures are the same magnification; the bar in Figure 2 is 40 μm long.

Segregation of a muscle determinant

Previous studies with cleavage-arrested embryos of *C. intestinalis* have shown that blastomeres of the muscle lineage in arrested embryos will eventually develop acetylcholinesterase (Whittaker, 1973). Similar experiments with *M. arenata*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produced the same results, at least in the earlier stages. Such observations support the theory that an autonomously acting cytoplasmic determinant of acetylcholinestrase development is being segregated in the muscle lineage cells.

Embryos of *M. arenata* were placed in 2 μg/ml cytochalasin B (Sigma) at various cleavage stages after fertilization. These were then reacted for acetylcholinesterase 8 to 9 hr after the 2-cell stage. The maximum cell numbers producing acetylcholinesterase and the relative positions of these cells in the embryo matched those of the known ascidian muscle cell lineage: one blastomere at 1-cell, two at 2-cell, two at 4-cell, two at 8-cell, and four at 16-cell. Figures 3–5 depict the last three of these stages.

A majority of the embryos at all of the various cleavage-arrested stages developed acetylcholinesterase in one or more of the muscle lineage blastomeres and a modest number of embryos at 2-, 4- and 8-cell stages produced the maximum lineage numbers of reacting cells (two). Interestingly, few embryos at the cleavage-arrested 16-cell stage produced enzyme in more than two cells, one on each bilateral side. Figure 5 illustrates one of the embryos in which all four of the muscle lineage cells produced acetylcholinesterase. Usually only the B5.1 cells synthesize acetylcholinesterase. This restriction to two rather than four cells is conceivably related to the lesion that results in a limited expression of acetylcholinesterase in *M. arenata*.

A limitation of expression seemed also to occur in later stages as well, but there the results were less certain. The chorion of *M. arenata* adheres closely to the embryo and appears to exert tension during development. Consequently, the acetylcholinesterase-producing muscle lineage cells in cleavage-arrested 32-cell and 64-cell stages tend to remain aggregated together in two bilateral groups. Aggregation combined with high yolk content of the cells and the large number of nuclei which accumulate make it essentially impossible to discern cell boundaries within groups of older myoblasts.

Acetylcholinesterase dependence on protein and RNA synthesis

The time at which enzyme was first detected histochemically (Fig. 1) is apparently the time of first acetylcholinesterase synthesis. When 200 μg/ml puromycin di-HCl (Sigma) was added to embryos of *M. arenata* 30 min before the time (at 6 hr) of first acetylcholinesterase staining no enzyme was detected histochemically at 8 to 9 hr time. Similarly, embryos placed in puromycin at

FIGURES 6–13. Embryos of *Ciona intestinalis* stained histochemically for succinic dehydrogenase.

FIGURE 6. Unfertilized egg.

FIGURE 7. 2-cell stage.

FIGURE 8. 4-cell stage.

FIGURE 9. 8-cell stage. Side view of the bilaterally symmetrical embryo.

FIGURE 10. 16-cell stage.

FIGURE 11. 32-cell stage.

FIGURE 12. 64-cell stage.

FIGURE 13. Middle tailbud stage at about the time of first melanocyte differentiation (12 hr development at 18° C).

The muscle lineage desginations are according to Ortolani (1955). All figures are the same magnification; the bar in Figure 13 is 40 μm long.

6 hr produced, even after many hours, only the slight amount of enzyme activity that would ordinarily be detected at 6 hr time. Puromycin at 200 μg/ml causes 95 to 99% inhibition of protein synthesis in ascidian embryos (Whittaker, 1973, 1977). Presumably the cytoplasmic factor being segregated is not a preformed inactive acetylcholinesterase since the occurrence of activity seems to depend directly on protein synthesis.

There was also an actinomycin D sensitivity period for acetylcholinesterase synthesis in this species. This occurred between 2 and 3 hr before the time of enzyme synthesis (Fig. 1). When embryos were treated continuously with 20 μg/ml actinomycin D (Sigma, Grade III) beginning at 3 hr after the 2-cell stage, no acetylcholinesterase developed subsequently. If treatment was started at 4 hr some slight amount of enzyme activity was found at hatching time. Progressively more enzyme activity was found the later after 4 hr that actinomycin D treatment was started. Since actinomycin D at this concentration produces a maximal inhibition of RNA synthesis in ascidian embryos (Smith, 1967; Mansueto-Bonaccorso, 1971), occurrence of acetylcholinesterase probably requires a specific embryonic period of RNA synthesis. Enzyme messenger RNA (mRNA) synthesis most likely occurs during this time. On the basis of other studies (Whittaker, 1977), one can assume that enzyme synthesis would be resistant to actinomycin D if a performed mRNA for the enzyme were present.

Mitochondrial segregation in urodele embryos

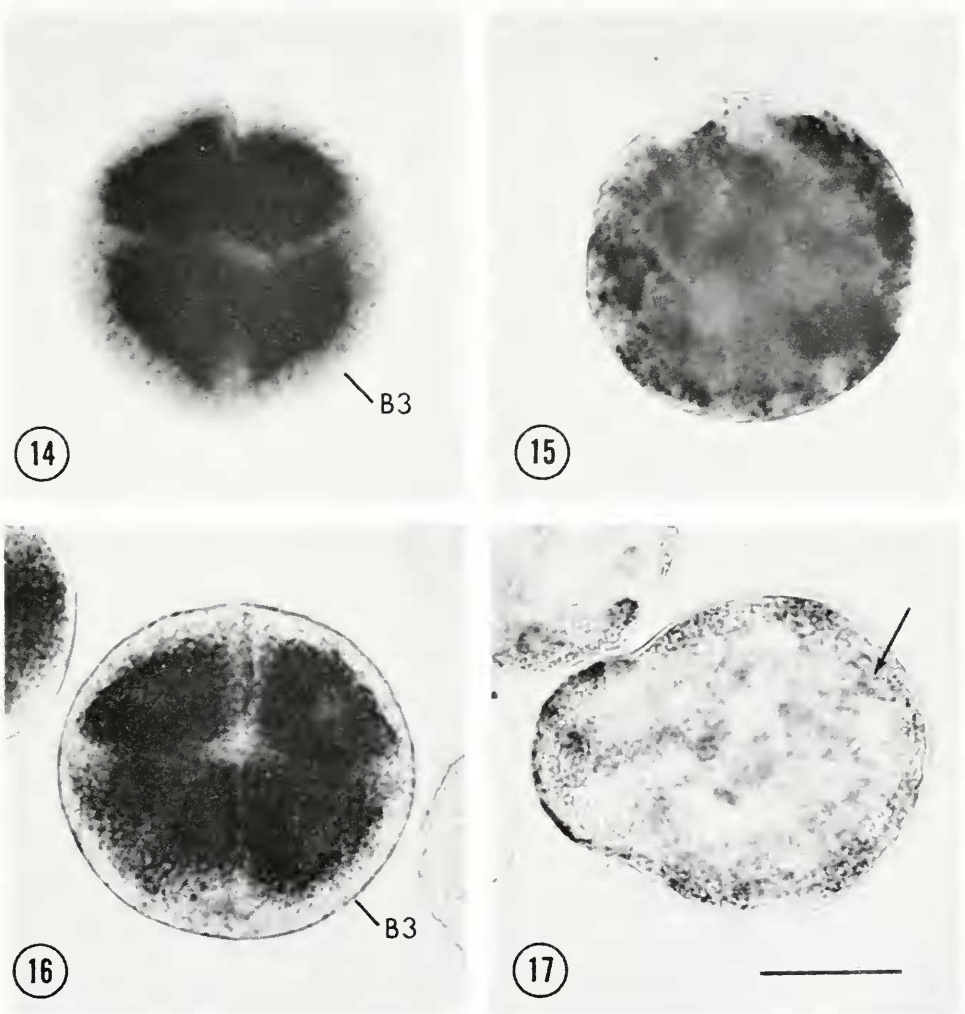
Succinic dehydrogenase reactions in early embryonic stages of *C. intestinalis* showed clearly the pattern of mitochondrial distribution in the embryos (Figs. 6–13). Before germinal vesicle breakdown, which occurs in the oviduct, mitochondria are distributed uniformly in the subcortical cytoplasm of the egg, as seen by the distribution of succinic dehydrogenase in cryostat sections (Patricolo, 1964). After germinal vesicle breakdown and before fertilization there is a migration of mitochondria to the vegetal half of the egg (Fig. 6). After fertilization they migrate further into the vegetal half of the egg and many of them eventually form a mitochondrial crescent, which is seen most clearly at the 2-cell stage (Fig. 7).

Although mitochondria were distributed elsewhere in the egg and embryos, a major concentration occurred in the muscle lineage cells, according to the lineage patterns established by Conklin (1905) and Ortolani (1955). One should note particularly the distribution of activity at the 4-cell stage (Fig. 8) where there is obviously segregation of much more activity into the B3 pair of blastomeres. At the tailbud stages one sees the strong localization of enzyme in the differentiating muscle cells of the tail (Fig. 13). Identical results were obtained with embryos of another species, *M. occidentalis*.

Similar enzyme distributions can be shown with a second mitochondrial enzyme, cytochrome oxidase. Histochemical localizations of this enzyme were identical in both *C. intestinalis* and *M. occidentalis* to those shown in Figs. 6–13 for succinic dehydrogenase. This duplication lends additional confidence to the likelihood that these staining reactions reveal the mitochondrial distribution rather than simply reactive peculiarities of a particular enzyme.

Mitochondrial enzymes in anural embryos

There was no evidence at any embryonic stage that mitochondria were differentially segregated into muscle lineage blastomers of *M. arenata*. Embryos were examined at cleavage stages and at various later developmental stages. At second cleavage the two posterior (B3) blastomeres are slightly larger than the



FIGURES 14–17. Embryos of *Molgula arenata* stained histochemically for the mitochondrial enzymes succinic dehydrogenase and cytochrome oxidase.

FIGURE 14. 4-cell stage embryo stained for succinic dehydrogenase.

FIGURE 15. Embryo 6 hr after the 2-cell stage stained for succinic dehydrogenase.

FIGURE 16. 4-cell stage embryo stained for cytochrome oxidase.

FIGURE 17. Hatching larva stained for cytochrome oxidase. The arrow indicates the position of one arm of the muscle cell crescent.

All figures are the same magnification; the bar in Figure 17 is 40 μm long.

anterior pair. Figure 14 shows that distribution of succinic dehydrogenase occurs in proportion to the size of the blastomeres. There is no obvious differential segregation of activity into the posterior pair. At the neurula stage (Fig. 15) the developing larva had no enzyme localization in those presumptive muscle rudiments that so clearly showed the presence of acetylcholinesterase in other experiments (Fig. 2). Cytochrome oxidase staining revealed the same result: no segregation of enzyme at either the 4-cell stage (Fig. 16) or in the hatching larva (Fig. 17). As described in detail elsewhere (Whittaker, 1979), the hatching larva emerges "head" end first from the chorion; in Fig. 17 the smaller bulge at the left is the emerging anterior end of the larva. The crescent of myoblasts is located at the position of the arrow. There is no differential accumulation of cytochrome oxidase staining in this region of the embryo, or elsewhere.

Discussion

Preformation of mitochondria in the ascidian oocyte is an apparent adaptation to the needs of rapid development in oviparous embryos. Presumably the embryo is unable to synthesize sufficient mitochondria during a brief development time to meet the high energy requirements of larval tail muscle, and must prepare some of these in advance of embryonic development. Ascidian embryos synthesize additional mitochondria during development. Mancuso (1962) noted bilobed mitochondrial structures especially in myoplasmic regions of the *Ciona* embryo, and concluded that muscle mitochondria multiply during embryogenesis. Measurements of mitochondrial enzyme activity indicate that only about half of the number of mitochondria found at larval hatching occur preformed in the fertilized egg (D'Anna and Metafora, 1965; D'Anna, 1966).

According to enzyme activity measurements by Berg (1956, 1957) on separated blastomeres at the 4-cell stage roughly two-thirds of the original mitochondria are segregated (in *Ciona*) into the two B3 muscle lineage blastomeres. A large proportion of these become actively segregated into the later cells of the muscle lineage. While mitochondrial preformation and segregation may be necessary for optimal physiological function of the larva at hatching, the important question is whether this initial concentration directly provides information in the developing muscle system.

Larvae of most ascidian species have a histospecific acetylcholinesterase that occurs in the tail muscle cells (Durante, 1956, 1959). One anural species, *M. arenata*, also developed enzyme in the presumptive muscle cells of the aborted tail (Whittaker, 1979), which indicates that these cells are unquestionably programmed to become muscle. Although myofibrils were not obvious, and probably did not occur, the cells nevertheless differentiated part of their histospecific acetylcholinesterase.

Evidence from experiments with cleavage-arrested embryos of *Ciona* (Whittaker, 1973) and microsurgically isolated partial embryos (Whittaker, Ortolani, and Farinella-Ferruzza, 1977) suggests that ascidian embryos localize and segregate a cytoplasmic determinant concerned with synthesis of acetylcholinesterase by the developing muscle cells. This factor appears to be neither the enzyme itself nor a preformed mRNA for acetylcholinesterase, and is probably an agent responsible

for activating particular genetic programs in the embryo (Whittaker, 1973). A similar determinant apparently occurs in *M. arenata*. Segregation of a determinant was demonstrated by cleavage-arresting embryos of *M. arenata* with cytochalasin B, and finding the eventual development of acetylcholinesterase in blastomeres of the ascidian muscle lineage pattern. Also, normal occurrence of acetylcholinesterase activity was blocked by puromycin and actinomycin D, suggesting thereby that there is neither preformed enzyme nor preformed mRNA for acetylcholinesterase.

Histochemical staining of the mitochondrial enzymes succinic dehydrogenase and cytochrome oxidase in embryos and larvae of *M. arenata* showed unequivocally that no "crescent" localization of mitochondria occurred after fertilization and no subsequent differential segregation of mitochondria took place. The conclusion that anural embryos lack a differential localization and segregation of mitochondria is thought to be valid for two reasons. Results with the same techniques in a normal urodele embryo series (Figs. 6–13) unambiguously show differential mitochondrial distribution. Secondly, concentrations of the histochemical reaction products follow the Beer-Lambert law, and the human eye is sufficiently sensitive to distinguish even minor concentration differences in the products.

In *M. arenata* there is an uncoupling of mitochondrial behavior and muscle cell differentiation. Mitochondria are not localized and not differentially segregated; yet there is determination of larval muscle and its partial differentiation. This shows without question that the mitochondrial accumulation in muscle cells of ascidian embryos has no direct informational relationship to the cellular differentiation. At the same time it is likely that the cytoplasmic determinants of muscle are not physically associated with the myoplasmic mitochondria. One cannot, however, rule out the possibility that the same physical mechanism is involved in both mitochondrial segregation and segregation of the determinants responsible for muscle differentiation. If so, the processes operate independently of one another.

The demonstration of disjunction between mitochondrial segregation and muscle differentiation is a potent example of the intrinsic superiority of nature's experiment. Theodore Boveri urged us long ago to seek the natural experiment where possible: "the investigator of living processes will make it his special concern to find out abnormalities, in which he has not intervened with his crude methods, where he can penetrate into the nature of the alteration" (Boveri, 1908, p. 216).

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Summary

The ascidian *Molgula arenata* produces an anural larva in which development of the tail and other urodele features has been suppressed. The embryos never-

theless developed part of the histospecific tail muscle acetylcholinesterase; the presumptive myoblasts have obviously acquired the muscle differentiation program. When cleavage-stage embryos were prevented from undergoing further division by treatment with cytochalasin B, acetylcholinesterase eventually developed in blastomeres of the muscle lineage. These anural embryos apparently segregate a cytoplasmic determinant concerned with acetylcholinesterase development into cells of the muscle lineage. In this species there was no localization and segregation of mitochondrial succinic dehydrogenase and cytochrome oxidase in the muscle lineage, as found in embryos of two urodele ascidians, *Ciona intestinalis* and *Molgula occidentalis*. The causal determinant of histospecific acetylcholinesterase expression is not, therefore, a differential localization of mitochondria nor is segregation of the muscle determinants linked directly to mitochondrial segregation.

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